MOLECULAR ONCOLOGY 9 (2015) 1852–1867

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PEA3 transcription factors are downstream effectors of Met signaling involved in migration and invasiveness of Met-addicted tumor cells

Zoulika Kherrouche[a], *Didier Monte*[b], *Elisabeth Werkmeister*[a,c], *Luc Stoven*[a], *Yvan De Launoit*[a], *Alexis B. Cortot*[a,d], *David Tulasne*[a,*,1], *Anne Chotteau-Lelievre*[a,1]

[a]*CNRS UMR 8161, Institut de Biologie de Lille, Institut Pasteur de Lille, Université de Lille, SIRIC ONCOLille, Lille 59021, France*

[b]*CNRS USR 3078, Institut de Recherche Interdisciplinaire, Université de Lille, Villeneuve d'Ascq 59658, France*

[c]*BioImaging Center Lille Nord de France, Lille 59021, France*

[d]*Thoracic Oncology Department, Lille University Hospital, Université de Lille, France*

ARTICLE INFO

Article history:
Received 25 February 2015
Received in revised form
16 June 2015
Accepted 1 July 2015
Available online 15 July 2015

Keywords:
PEA3
Transcription factor
ETS
Met
EGFR
Receptor tyrosine kinase
Migration
Lung tumorigenesis

ABSTRACT

Various solid tumors including lung or gastric carcinomas display aberrant activation of the Met receptor which correlates with aggressive phenotypes and poor prognosis. Although downstream signaling of Met is well described, its integration at the transcriptional level is poorly understood. We demonstrate here that in cancer cells harboring *met* gene amplification, inhibition of Met activity with tyrosine kinase inhibitors or specific siRNA drastically decreased expression of ETV1, ETV4 and ETV5, three transcription factors constituting the PEA3 subgroup of the ETS family, while expression of the other members of the family were less or not affected. Similar link between Met activity and PEA3 factors expression was found in lung cancer cells displaying resistance to EGFR targeted therapy involving *met* gene amplification. Using silencing experiments, we demonstrate that the PEA3 factors are required for efficient migration and invasion mediated by Met, while other biological responses such as proliferation or unanchored growth remain unaffected. PEA3 overexpression or silencing revealed that they participated in the regulation of the MMP2 target gene involved in extracellular matrix remodeling. Our results demonstrated that PEA3-subgroup transcription factors are key players of the Met signaling integration involved in regulation of migration and invasiveness.

Abbreviations: Met, hepatocyte growth factor receptor; PEA3, polyomavirus enhancer activator 3; ER81, ETS related 81; ERM, ETS related molecule; ETS, E-twenty-six; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; RT-PCR, reverse transcription PCR; SD, standard deviation; siRNA, small interfering RNA.

* *Corresponding author*. CNRS UMR 8161, Institut de Biologie de Lille, Institut Pasteur de Lille, 1 Rue Pr Calmette, BP447, 59021 Lille, France.

E-mail address: david.tulasne@ibl.cnrs.fr (D. Tulasne).

[1] The authors contributed equally to the study.

http://dx.doi.org/10.1016/j.molonc.2015.07.001

1. Introduction

The receptor tyrosine kinase Met is expressed predominantly in cells of epithelial origin. Its high affinity ligand is the hepatocyte growth factor/scatter factor (HGF/SF) (Birchmeier et al., 2003). HGF/SF and Met are essential to embryonic development, notably for organization of epithelial organ, and in adult for tissue regeneration like liver or epidermis (Bladt et al., 1995; Borowiak et al., 2004; Chmielowiec et al., 2007; Huh et al., 2004; Schmidt et al., 1995; Uehara et al., 1995). Upon ligand binding and subsequent Met dimerization, several tyrosine residues in the intracellular domain become phosphorylated, allowing recruitment of cytoplasmic proteins involved in activating multiple intracellular signaling pathways (Ponzetto et al., 1994). Integration of this signaling network allows induction by ligand-activated Met of various biological responses in epithelial cell lines, including proliferation, survival, migration, invasion or morphogenesis.

In many cancers, uncontrolled Met activation is observed and is associated to tumorigenesis and metastasis. A direct link between Met and cancer has been evidenced by characterization of germline activating mutations in patients affected by hereditary papillary renal carcinoma (Schmidt et al., 1997). In a significant number of human cancers, HGF/SF and Met are found overexpressed (Comoglio et al., 2008). For instance in lung and gastric cancers, Met overexpression is observed in about half of tumors. This overexpression is the consequence of various mechanisms, including the activation of other oncogenes such as RAS, the expression of transcription factors such as Pax 5, HIFα or ETS1, or the repression of miRNA targeting Met sequence (Gambarotta et al., 1996; Hwang et al., 2011; Ivan et al., 1997; Kanteti et al., 2009; Kubic et al., 2014; Pennacchietti et al., 2003). Overexpression of Met can also result from an increase of *met* gene copy number through chromosome 7 polysomy and *met* amplification, which are found in about 5–20% of the gastric and lung cancers (Lee et al., 2012; Tsuta et al., 2012). This gene amplification can trigger high level of Met expression leading to spontaneous dimerization and subsequent Met tyrosine kinase activation (Ponzetto et al., 1991). Cancer cell lines harboring *met* gene amplification and subsequent ligand-independent activation displayed addiction to Met signaling, since inhibition of Met expression or activity leads to decreased cell growth and survival (Corso et al., 2008).

Interestingly, in non-small cells lung carcinoma, resistance to inhibitors targeting mutated Epidermal Growth Factor Receptor (EGFR) involves amplification of the *met* gene observed in 5–20% of the patients (Engelman et al., 2007). This leads to strong Met overexpression and its ligand-independent activation, which shortcuts inhibition of EGFR activity through activation of similar downstream signaling pathway including RAS–ERK and PI3K–AKT pathways (Bertotti et al., 2009; Wagner et al., 2013). Similar mechanism of resistance has been recently revealed in colorectal cancers treated with anti-EGFR antibody (Bardelli et al., 2013).

Although the intracellular signaling network downstream of Met is well described, the integration of the signal at the transcriptional level is still poorly understood. Nevertheless, Met signaling is able to regulate activity or expression of several transcription factors including STAT3, ETS1, NFκB or p53 (Boccaccio et al., 1998; Fan et al., 2005; Furlan et al., 2012; Paumelle et al., 2002). In oral squamous carcinoma, HGF/SF stimulation is also able to trigger expression of the ETV4 (Pea3) transcription factor (Hanzawa et al., 2000). Interestingly, we have previously shown that Met and ETV4 display similar pattern of expression during branching morphogenesis of epithelial organs such as lung, kidney and mammary gland (Andermarcher et al., 1996; Chotteau-Lelievre et al., 1997; Sonnenberg et al., 1993). In addition, we showed that overexpression of ETV4 and ETV5 (Erm) in mammary epithelial cells promote branching morphogenesis in matrix, similar to those induced by HGF/SF (Chotteau-Lelievre et al., 2003). In spinal cord, expansion of the ETV4 positive pool of motor neurons is in part dependent on Met signaling induced by HGF/SF (Caruso et al., 2014; Helmbacher et al., 2003).

The PEA3 group of transcription factors is composed by three members, ETV4 (Pea3), ETV5 (Erm) and ETV1 (ER81), which display high sequence homology and similar subdomain organization. The three PEA3 members belong to the larger ETS family of transcription factors, all homologous on their DNA binding domain (ETS binding domain) (de Launoit et al., 2006). The 27 members of the ETS family are divided in 5 or 12 subgroups according to their preferential DNA binding sequence or their homology in their DNA binding domain (Laudet et al., 1999; Wei et al., 2010). Expression of PEA3 factors overlaps in many organs during embryonic development and in adult. However their expression can be drastically different in certain tissues. This suggests both overlapping and distinct functions of the three members according to the developmental process. For instance knockout mouse models demonstrate that ETV4 and ETV5 are both involved in kidney development (Lu et al., 2009), while ETV1 plays a major role in motor coordination (Arber et al., 2000).

Deregulation of the expression of PEA3 factors is associated with carcinogenesis. In Ewing's sarcoma and prostate cancers, aberrant activation or overexpression is due to a chromosomal translocation (Hollenhorst et al., 2011; Urano et al., 1996). PEA3 factors are also found overexpressed in other cancers such as breast, gastric, ovarian or lung cancers (Oh et al., 2012). In most cases the overexpression modifies proliferating, migrating and/or invading properties that is part of the tumorigenic and/or metastatic coming. As a consequence, the deregulation of PEA3 target genes has a key role in these processes. So far, few PEA3 target genes involved in the regulation of these biological responses have been described. These include matrix metalloproteases such as MMP2, MMP7 and MMP9, transcription factors such as Twist1 or Snail, or factors such as Cyclooxygenase-2, Bax or Cyclin D2 (Firlej et al., 2005; Howe et al., 2003; Ladam et al., 2013; Qin et al., 2008, 2009; Subbaramaiah et al., 2002; Yamamoto et al., 2004). Several data, focalized on gastric or lung carcinoma, reported the role and importance of PEA3 factors in the progression of the cancer and in association with expression of markers of epithelial to mesenchymal transition or MMPs (Boedefeld et al., 2005; Hakuma et al., 2005; Hiroumi et al., 2001; Li et al., 2011; Sloan et al., 2009; Yamamoto et al., 2004).

We demonstrate here that in gastric and lung cancerous cells harboring *met* gene amplification, expression of the

PEA3 transcription factors is dependent on Met activity. The three factors are required for efficient migration and invasion triggered by Met in these cells, while other biological responses are not affected. These results demonstrate that in Met-addicted cells integration of the Met signaling at the transcriptional level involved a subgroup of transcription factors regulating specifically migration and invasion.

2. Materials and methods

2.1. Cytokines, drugs, and cell cultures

Human recombinant HGF and EGF were purchased from Peprotech (Rocky Hill, NJ, USA). The Met kinase inhibitor PHA-665752 and MEK inhibitor U0126 were purchased from Promega (Madison, WI, USA), the PI3K inhibitor LY294002 from BioMol (Germany), Anisomycin, DMSO, Hoechst-33258 dye and mitomycin-c were obtained from Sigma (St Louis, MO, USA), DilC12 fluorescent dye was purchased from BD and Gefitinib from Santa cruz.

Cells were cultured in Invitrogen media supplemented with 10% fetal bovine serum (FBS, Invitrogen) and 1% antibiotics (penicillin 10,000 U/ml–streptomycin 10,000 μg/ml, Invitrogen). The human gastric carcinoma cell lines GTL16 (a gift from Dr. Silvia Giordano, Institute for Cancer Research and Treatment, University of Turin, Italy) and Hs746T (A.T.C.C. number HTB-135), the breast cancer cell line MDA-MB231 (A.T.C.C. number HTB-26) and the cervical cancer Hela(0) cells (A.T.C.C. number CCL-2) were cultured in DMEM; the lung adenocarcinoma HCC827 cell line and its *met* amplified counterpart HCC827-GR6 (a gift from Pr. P.A. Jänne, Dana-Farber Cancer Institute, Boston, MA, U.S.A.) (Engelman et al., 2007) were cultured in RPMI 1640; the lung cancer cell lines EBC-1 (Japanese Collection of Research Bioresources Cell Bank, JCRB0820) and A549 (A.T.C.C. number CCL-185) were cultured in EMEM and Ham's F-12K (Kaighn's) medium respectively. MCF-10A (A.T.C.C. number CRL-10317) spontaneously immortalized human mammary epithelial cells were cultured in DMEM and HAM's F-12 vol/vol (Invitrogen) supplemented with 5% horse serum (Invitrogen), 500 ng/ml hydrocortisone (Calbiochem), 20 ng/ml EGF, 10 μg/ml insulin (Sigma), and 100 ng/ml cholera toxin (Calbiochem) and 1% antibiotics. Cells were cultured at 37 °C in a water-saturated 5% CO_2 atmosphere.

2.2. Nuclear extracts

Cells were treated 5 h with 25 μM of the proteasome inhibitor ALLN (Calbiochem), collected in five volumes of 10 mM Tris pH7.5, 140 mM NaCl, 1× complete protease cocktail inhibitor (Roche), pelleted and suspended in three volumes of the hypotonic buffer (10 mM Tris pH7.5, 25 mM KCl, 2 mM magnesium acetate, 1 mM DTT, 1× cocktail inhibitor). After 10 min of incubation at 4 °C, cells were disintegrated using a Dounce homogenizer. Nuclei were collected by centrifugation and suspended in one volume of 10 mM Tris pH7.5, 350 mM KCl, 2 mM magnesium acetate buffer and 1× cocktail inhibitor. Cells debris were removed by centrifugation and the supernatant dialyzed 2 h in 50 mM HEPES pH7.5, 20% glycerol, 50 mM KCl, 2 mM EGTA, 4 mM $MgCl_2$, 1 mM PMSF. The nuclear extracts were stored at −80 °C.

2.3. Western blot analysis and antibodies

Cells were lysed in RIPA buffer containing 25 mmol Tris HCl (pH7.6), 150 mmol NaCl, 1% Nonidet P-40, 1% sodium deoxycholate, 0.1% SDS, 20 mmol b-glycerophosphate, 1 mmol sodium orthovanadate, 1 mmol sodium fluoride, 1 mmol phenylmethylsulfonyl fluoride, 1 mg/mL aprotinin and 1 mg/mL leupeptin. Protein concentrations were determined with the BCA Protein Assay Kit (Thermo Scientific Pierce). Cell lysates containing an equal amount of total protein were separated on SDS-polyacrylamide gel electrophoresis (10%) or NuPAGE Bis-Tris gels (4%–12%; Invitrogen) at 130 V for 2 h, and proteins were transferred at 100 V for 1.5 h on Protran Nitrocellulose membranes (Whatman). Prestained broad-range molecular mass markers (New England BioLabs) were used as standards in each gel. Protein detection was performed using the ECL Western blotting detection Reagent (GE-Healthcare, Amersham) or SuperSignal West Femto (ThermoScientific) when required. The following antibodies were used at a concentration of 1:1000 unless otherwise indicated: Met (Cell Signaling, 3148), phospho-Met (Cell Signaling, 3126), EGFR (Cell Signaling, 2646), phospho-EGFR (Cell Signaling, 2236), AKT (Santa Cruz, sc-8312), phospho-AKT (Ser473) (Cell Signaling, 9271), ERK (Santa Cruz, sc-154), phospho-ERK (Thr202/Tyr204) (Cell Signaling, 9106), ERM/ETV5 antibody (anti-ERM^{12-226}) (Baert et al., 1997), ER81/ETV1 (Abcam, ab81086, 1/500), PEA3/ETV4 (Santa Cruz, sc-113), ETS1 (Santa Cruz, sc-111), ERG (Epitomics, 2085), Actin (Santa Cruz, sc-47778, 1:250), GAPDH (Santa Cruz, sc-32233).

2.4. Plasmid constructs

The pcDNA3.2-Flag vectors encoding ETV1, ETV4 and ETV5 were constructed as follows. Human fragments were amplified by PCR with the primers listed in Table S1 and inserted into the pcDNA3.2-Flag plasmid between the BamHI and XbaI restriction sites. The pCAGGs empty vector and the full length human Met expressing vector, Met-pCAGGs, were previously described (Foveau et al., 2009).

2.5. Transfections

GTL16 cells were plated on 6-well plates (400,000 per well) and transfected the next day with JetPrime reagent (Polyplus Transfection, Illkirch, France; 2 μg DNA/4 μl JetPrime reagent/200 μl JetPrime buffer in 2 mL complete medium). After 4 h, the transfection medium was replaced by complete medium.

2.6. RNA interference

For silencing, 300,000 cells were incubated with 4.5 μL Lipofectamine 2000 (Invitrogen) mixed with 120 nM of a pool of Stealth siRNAs (Invitrogen) targeting the genes of interest. The cells were then plated in a 6-well plate in a final volume of 1.5 mL of complete medium. The same batch of transfected cells were trypsinised after 24 h and divided in a 12-wells plate

for proliferation or cell death analysis and a 6-wells plate dedicated to RNA extraction. After an additional 24 h culture, siRNA efficiencies were checked by RT-qPCR.

The following siRNA were purchased from Life technology: Control Stealth siRNA negative control, a set of three distinct Stealth siRNA targeted ETV1 [5′-CCCUACAACGAAGGCUACG UGUAUU-3′, 5′-CACCAAUAGUCAGCGUGGGAGAAAU-3′, 5′-GG AGAGAGAUAUGUCUACAAGUUUG-3′], ETV4 [5′-GGGCAGAGC AACGGAAUUU-3′, 5′-GGACUUCGCCUACGACUCA-3′, 5′-GAAU GGAGUUCAAGCUCAU-3′], ETV5 siRNA [5′-CCGAUUAUAC UUUGACGACACUUGU-3′, 5′-GGACACAGAUCUGGCUCACGAU UCU-3′, 5′-CCAUCGGCAAAUGUCAGAACCUAUU-3′] and Met [5′-CCAUUUCAACUGAGUUUGCUGUUAA-3′, 5′-UCCAGAAGA UCAGUUUCCUAAUUCA-3′, 5′-CCGAGGGAAUCAUCAUGAAA-GAUUU-3′]. Each experiment was repeated at least three times.

2.7. Real-time RT-PCR

Total RNA was extracted from the cells 24 h after pharmacological treatments or 48 h after DNA or si-RNA transfections using the Nucleospin RNA/Protein Kit (Macherey–Nagel) according to the manufacturer's protocol. Total cDNA was reverse-transcribed from the total RNA with random hexamers using the High Capacity cDNA Reverse Transcriptase Kit (Applied Biosystems). Analysis of transcript relative fold copy number was carried out by quantitative real-time PCR using Fast SYBR Green mix (Applied Biosystems) in an MX3005P instrument (Stratagene) as described by the supplier, with specific primers listed in Table S1. The relative expression was calculated using the $2^{-\Delta\Delta CT}$ method. The mRNA levels of each target gene were normalized to the levels of the housekeeping B2M gene and presented as fold induction relative to the control DMSO or control siRNA. Results were represented as means with error bars showing standard deviations of at least three independent experiments.

2.8. Cell proliferation and cell death

For proliferation analysis, cells were plated in triplicate in 12-well plates at 20,000 per well in a final volume of 1 mL of medium. At each time point, cells were washed with PBS, trypsinised, and counted with a cell counter (Z1 Beckman Coulter). Results were represented as means ± SD from triplicate samples from one of three representative experiments.

Cell death was assessed 48 h after siRNA transfection on cells treated 4 h with DMSO or 50 μM anisomycin. Annexin V and Propidium Iodide staining was performed using Tali apoptosis kit according to the manufacturer and analyzed on a Tali apparatus (Life Technology). Representative experiment showing percentage of unstained, annexin V and PI staining, is presented.

2.9. Migration and invasion assays

Migration of cells was assayed in Transwell cell culture chambers with a polycarbonate filter-membrane of 8 μm-pore size in 24-well plates (BD Biosciences). Cells were seeded to the upper compartment of the chamber at a cell density of $2 \cdot 10^5$ in 100 μl of serum-free medium. The lower chamber was filled with 10% FBS-containing medium. After 24 h of incubation at 37 °C, the non-migrating cells were removed by wiping with a cotton swab. The cells on the lower surface of the membrane were fixed with PFA and stained with Hoechst-33342. The number of migrated cells was counted using Image J software on images created on an AxioImagerZ1 Apotome microscope and the mean was determined. A migration assay was also performed in the presence of mitomycin-c (3 μg/ml) to inhibit cell proliferation. Invasion of cells, labeled with DiIC(12), was assayed in Fluoroblok invasion assay (BD Biosciences) and quantified using a FLUOstar OPTIMA reader (BMG Labtech).

2.10. Live cell microscopy

Microscopy was performed on a Zeiss AxioObserverZ1 fluorescence microscope equipped with a 5× Fluar lens (numerical aperture [NA] 0.25), an Axio mRm camera, a Lambda DG4 excitation source (Sutter Instrument Company), a DsRed Filter (Zeiss Filter Set 43HE) and an incubation Unit (XL Unit, Pecon). For time-lapse microscopy, the interior of the incubator surrounding the entire microscope system was heated to 37 °C and the CO_2 level was regulated to 5%. Cells were labeled with DilC12 (15 μg/ml; Becton Dickinson) and seeded in Ibidi 15 μ slide 8 wells (GmbH, München, Germany). Images were acquired with the Zen software (Zeiss) at a rate of 1 image per time interval of 10 min during 20 h. The Plugin MTrack2 implemented in the Fiji software (NIH) enabled to obtain the trajectory (xy-path) of each cell. Cell migration was assessed as the total path length in micrometers traveled and cell velocity (microns/h) was determined using Matlab (Mathworks). For statistical analysis, the Kruskal–Wallis test was applied under R environment.

2.11. Anchorage independent growth assays

A bottom layer of 0.5 mL of 0.4% agarose diluted in complete media was deposited onto 12-well plates. Transfected cells (4000/well) were added directly to a 0.6% agarose in media mixture and overlaid onto the bottom layer. Once solidified, media was added and cells grown for 10 days in a 37 °C incubator with 5% CO_2. Media was changed every 2–3 days. Colonies were photographed and counted under a standard light microscope. Results were illustrated with representative fields of clones and the means of three wells in three independent experiments ±SD.

2.12. Statistical analysis

Unpaired Student's T-test analysis was performed when sample groups were compared with a control group. p-Value <0.05 was considered statistically significant. Error bars represent the SD.

3. Results

3.1. Met activity is required for PEA3 expression in cells harboring met gene amplification

The gastric adenocarcinoma cells GTL16 and the non-small cell lung cancer (NSCLC) cells EBC-1 harbor a *met* gene

amplification leading to a constitutive activation of the receptor. When treated for 24 h to the Met tyrosine kinase inhibitor, PHA-665752 (PHA), quantitative RT-PCR experiments demonstrated that Met inhibition merely abolished mRNA expression of the three PEA3 factors, ETV4, ETV5 and ETV1 (Figure 1A). Western Blot analysis confirmed that both cell lines displayed constitutive Met phosphorylation that was abolished by PHA treatment (Figure 1B).

A similar inhibition of PEA3 members expression was obtained in response to Met inactivation in the gastric carcinoma cell line Hs746T exhibiting a splice site mutation of exon 14 with *met* locus amplification (Supplementary Figure S1A). In contrast, PEA3 expression is unaffected by Met inactivation neither in MCF10A, an immortalized non-tumorigenic mammary cell line (Figure S1B), nor in other cancerous cell lines A549, MDA-MB231 and Hela(0), which do not display *met* gene amplification or Met activation (Supplementary Figure S1C).

Expression of 13 other ETS factors, covering the 12 subgroups of the ETS family (Laudet et al., 1999), was evaluated in response to the Met inhibitor PHA-665752 in GTL16 and EBC-1 cells. The transcripts of the three PEA3 members drastically decreased upon Met inactivation (Figure 1C and D). Transcripts of ETS factors Ets1, Ets2 and Net displayed around 30–50% decreased expression whereas expression of the other ETS genes tested was mainly unaffected or even upregulated by PHA treatment. Western blot analysis on nuclear extracts confirmed that Met kinase inhibitor leads to decrease of ETV4, 5 and 1 expression, while ERG and ETS1 protein expression was unchanged (Figure 1D). It is worth noting that cells were treated with proteasome inhibitor to stabilize PEA3 factors such as ERM which has been shown to be degraded by proteasome (Baert et al., 2010). ETV1, ETV4 and ETV5 expression was also drastically inhibited by Met silencing with siRNA directed against Met compared to control siRNA (Figure 2A). Effective knockdown of Met was confirmed by quantitative RT-PCR (Figure 2B) and Western blotting (Figure 2C).

In order to determine whether PEA3 transcription factors are able to regulate in mirror Met expression, silencing of the three PEA3 were performed in GTL16 and EBC-1 cells. The efficiency of each pool of three siRNA targeting ETV1, 4 and 5 or the three pools together, were validated by strong decrease of their respective target or by decrease of the three PEA3 when the combination was used (Supplementary Figure S2). In contrast, Met expression was not decreased in response to PEA3 knock-down expression (Figure 2B and Supplementary Figure S2). This suggests that in tumor cell lines displaying *met* gene amplification, expression of the three PEA3 transcription factors are dependent of Met expression and activity, while they are not involved in regulation of Met expression.

In order to evaluate involvement of Met downstream signaling pathways on PEA3 members expression, cells were treated with the U0126 MEK inhibitor or the LY294002 PI3K inhibitor and PEA3 mRNA expression were determined. As shown in Figure 3A, inhibition of MEK activity decreased expression of the three *PEA3* genes while inhibition of PI3K did not. AKT and ERK phosphorylation was efficiently decreased by PI3K and MEK inhibitors respectively and both pathways were inhibited by PHA, confirming Met involvement in their activation (Figure 3B). These results suggest an involvement of MEK–ERK MAPK signaling in the regulation of PEA3 members expression. Nevertheless in response to MEK inhibitor, Met expression was also decreased. To rule out the possible involvement of Met downregulation on PEA3 expression, Met was overexpressed by transient transfection with a full length human Met expressing vector (Met-pCAGGs) in the presence or not of U0126. As expected, ectopic expression of Met was not affected by U0126 treatment. However, the mRNA levels of PEA3 factors were not restored by Met re-expression in the presence of the MEK inhibitor. This confirms that regulation of ETV1, ETV4 and ETV5 expression by Met involves the MEK–ERK signaling.

3.2. *PEA3 factors expression is dependent on both EGFR and Met in TKI EGFR resistant cells*

Resistance to EGFR targeted therapy in patients harboring EGFR mutation involves *met* gene amplification in 5–20% of the cases, with Met overexpression rescuing survival in response to EGFR inhibitors. In order to study Met-dependent expression of PEA3 members in the context of resistance to EGFR inhibitors, we used HCC827 cells, a lung adenocarcinoma cell line harboring a deletion in exon 19 of EGFR, and their derived cells HCC-GR6, displaying resistance to EGFR inhibitor through *met* gene amplification. As previously described, Western blot analysis showed that HCC827 cells displayed EGFR and Met basal phosphorylation, which was inhibited by EGFR inhibitor gefitinib (Engelman et al., 2007). Met phosphorylation was inhibited by PHA that did not affect EGFR activity (Figure 4A). This demonstrates that, in HCC827, EGFR is constitutively activated and regulates in turn Met activation. In HCC-GR6 cells, Met receptor was overexpressed and displayed a higher phosphorylation level compared to HCC827 cells. PHA and gefitinib inhibited their respective target but inhibited only slightly EGFR and Met respectively. Strong inhibition of both Met and EGFR was obtained by a PHA plus gefitinib co-treatment. Therefore in HCC-GR6, *met* gene amplification leads to its autonomous activation that can in turn regulate EGFR activity (Figure 4A).

We showed that, similarly to Met, ETV1, ETV4 and ETV5 transcripts were more expressed in the HCC-GR6 resistant cells than in the HCC827 parental cells (Figure 4B). In HCC827 cells, gefitinib inhibition decreased mRNA expression of ETV1, 4 and 5, while PHA did not, demonstrating that in this cell line expression of PEA3 factors is dependent of EGFR activity (Figure 4C). In contrast, in HCC-GR6, treatment with PHA-665752 or gefitinib alone moderately reduced PEA3 mRNA expression (between 25 and 70%). However, co-treatment with PHA or gefitinib abolished it, indicating that in HCC-GR6 expression of PEA3 factors is dependent of both EGFR and Met activity (Figure 4C). This was confirmed by Western blot against ETV1 and ETV5 (Figure 4A). The poor quality of the available antibodies directed against ETV4 did not allow efficient detection. This result demonstrates that *met* gene amplification in EGFR inhibitor resistant cells induces overexpression of PEA3 factors that is dependent on both EGFR and Met activities. Interestingly, the decrease of ETV1, 4 and 5 in response to gefitinib in HCC827 cells was rescued by treatment with Met ligand HGF/SF (Supplementary Figure S3A).

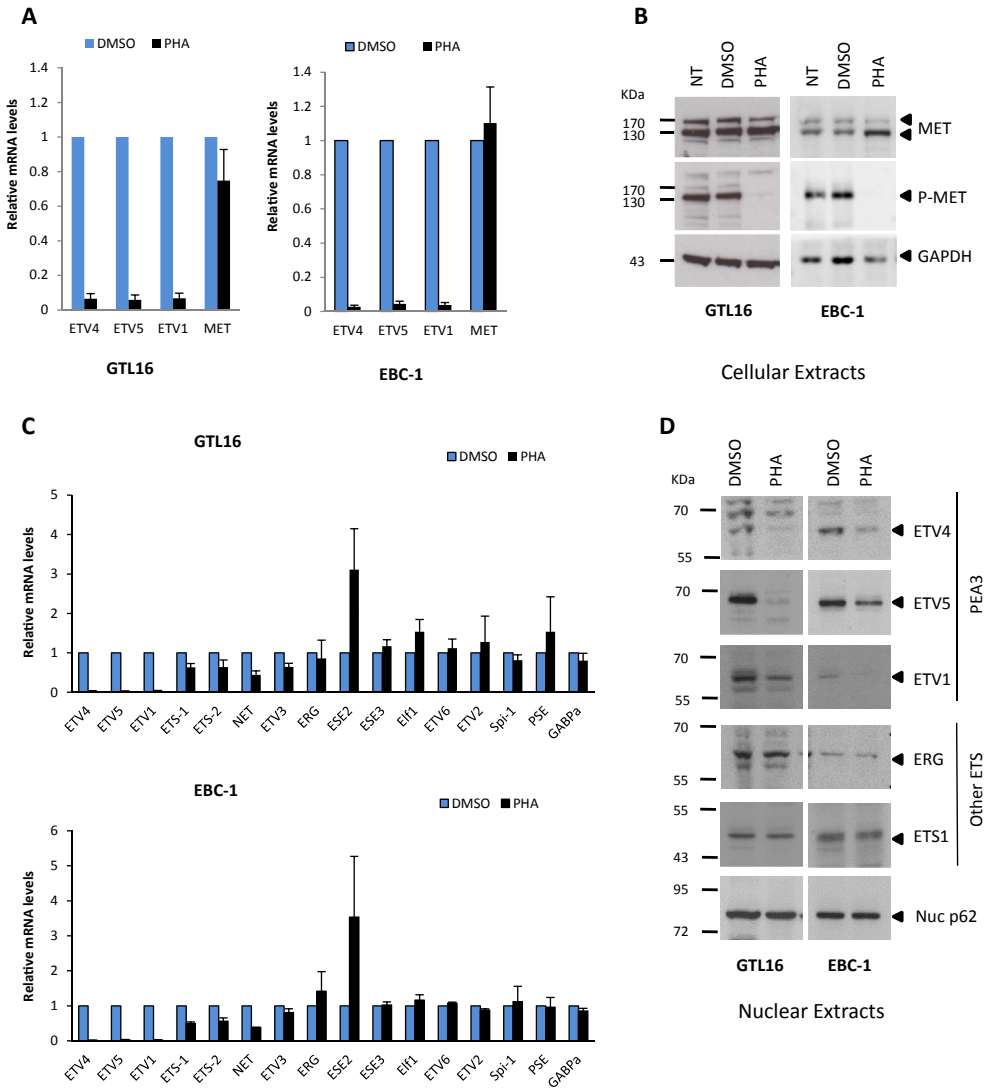

Figure 1 – Effect of Met tyrosine kinase inhibitor on PEA3 expression in Met addicted cells. (A) GTL16 and EBC-1 cancer cell lines harboring *met* gene amplification were treated for 24 h with 0.4 μM of Met tyrosine kinase inhibitor PHA-665752. mRNA expression of ETV4 (PEA3), ETV5 (ERM) and ETV1 (ER81) were measured by quantitative RT-PCR (RT-qPCR). The results presented are an average of at least three independent experiments with error bars showing standard deviations. (B) Western blot analysis was performed to determine the Met and phosphorylated Met protein levels in the whole cell lysate. GAPDH was used as a loading control. Results are representative of three experiments. (C) In GTL16 and EBC-1 cells treated with PHA-665752 mRNA expression of 16 ETS transcription factors was measured by quantitative RT-PCR. The mRNA levels of each target gene were presented as fold induction relative to the control DMSO and represented as means ± SD of at least three independent experiments. (D) Nuclear extracts of GTL16 and EBC-1 cell lines were analyzed by Western blotting using antibodies directed against of ETV4, ETV5, ETV1, ERG and ETS-1. Nuclear protein Nup-62 was used as a loading control. Arrow heads indicate positions of the proteins targeted by the respective antibodies.

Reversely, the decrease of ETV1, 4 and 5 in response to PHA in GTL16 cell line was rescued by EGF (Supplementary Figure S3B). These data confirm that Met or EGFR downstream signaling can rescue PEA3 factors expression.

3.3. *PEA3 factors are involved in the regulation of Met-induced migration and invasion*

Because, PEA3 factors expression is dependent on Met activity, we searched to evaluate their involvement in biological responses driven by Met. We compared the consequences of the silencing of Met and PEA3 factors in GTL16 and EBC-1 cell lines. As expected, Met silencing strongly abolished GTL16 and EBC-1 proliferation (Figure 5A) and colony formation in soft agar (Figure 5B). In addition, annexin V and PI staining showed that Met silencing increased cell death of both cell lines pretreated or not with anisomycin, an apoptotic inducer (Figure 5C). By contrast, silencing of the three PEA3 factors did not modify these responses, suggesting that these transcription factors are not involved in proliferation, unanchored growth and survival triggered by Met (Figure 5A–C).

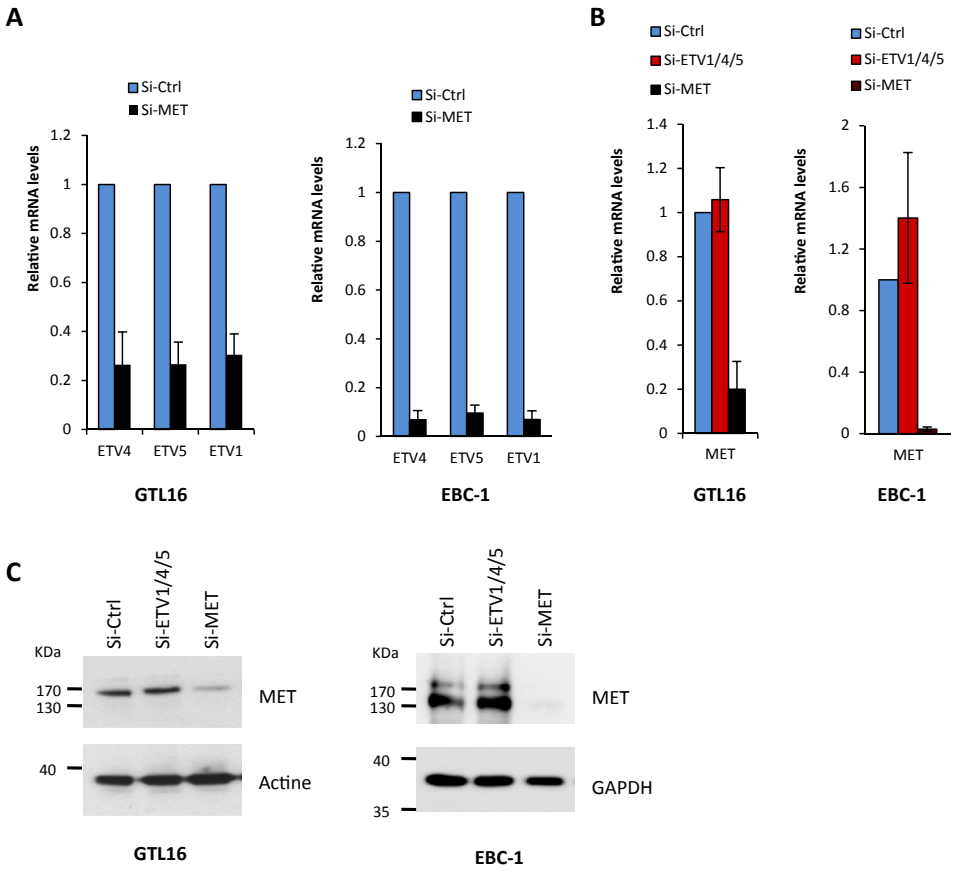

Figure 2 – Effect of Met knockdown on PEA3 factors expression. (A) Expression levels of ETV4, ETV5 and ETV1 mRNA were measured by RT-qPCR in GTL16 and EBC-1 cells after Met knockdown by specific siRNA. (B) The expression of Met mRNA was detected using RT-qPCR after PEA3 or Met knockdown by specific siRNA. (C) The Met inhibition following 48 h of siRNA knockdown was validated by Western blotting using an anti-Met antibody. GAPDH or actin was used as a loading control.

To evaluate the involvement of Met and PEA3 in migration, GLT16 and EBC-1 were cultured in Boyden chamber following the silencing of their expression. Met siRNA reduced by about 60% and almost completely GTL16 and EBC-1 migration, respectively (Figure 6A). Interestingly, silencing of the three PEA3 factors also inhibited migration of both cell lines to a lesser extent (between 30 and 40%). Measurement of individual cell motility by video-tracking confirmed the effect of Met and PEA3 silencing on cell migration (Figure 6B). Similarly, invasion through Boyden chambers coated with Matrigel was reduced by about 20% in GTL16 cells knocked-down for PEA3 or Met (Figure 6C). While inhibition of migration and invasion upon Met silencing could be the consequence of reduced proliferation and cell survival, this may not be the case for PEA3 factors silencing since it did not affect these responses (Figure 5).

Since PEA3 factors are known to regulate expression of various metalloproteinases (MMP) involved in cell migration and invasion, we evaluated their expression upon Met and PEA3 silencing. We found that both PEA3 and Met knockdown resulted in down-regulation of the matrix metalloproteinase-2 gene (MMP2; also known as type IV collagenase and gelatinase A), while PEA3 silencing did not significantly affect other MMPs including MMP1, MMP7 and MMP14 and urokinase-type plasminogen activator uPA (Figure 7A and B). Conversely, overexpression of PEA3 transcription factors was sufficient to increase expression of the MMP2 mRNA in GTL16 (Figure 7C and D). We also found that HCC-GR6 expressed much higher MMP2 mRNA that their HCC827 parental cells (Figure 7E). Accordingly to results obtained in GTL16, PEA3 factors silencing decreased MMP2 mRNA expression (Figure 7F).

4. Discussion

Amplification of *met* gene is found in 5–20% of the gastric and lung cancers (Lee et al., 2012; Tsuta et al., 2012). In derived cell lines from these cancers, *met* gene amplification was also observed. This is the case for the lung squamous cell carcinoma EBC-1 cells and for the gastric GTL16 cells harboring 11 *met* gene copies. In these cells, *met* gene amplification induces strong receptor overexpression leading to its ligand-independent activation (Ponzetto et al., 1991). These cell lines are addicted to Met activity since its silencing or tyrosine kinase activity inhibition induces growth arrest and finally cell death. Consistently, when xenografted in mouse, their tumor

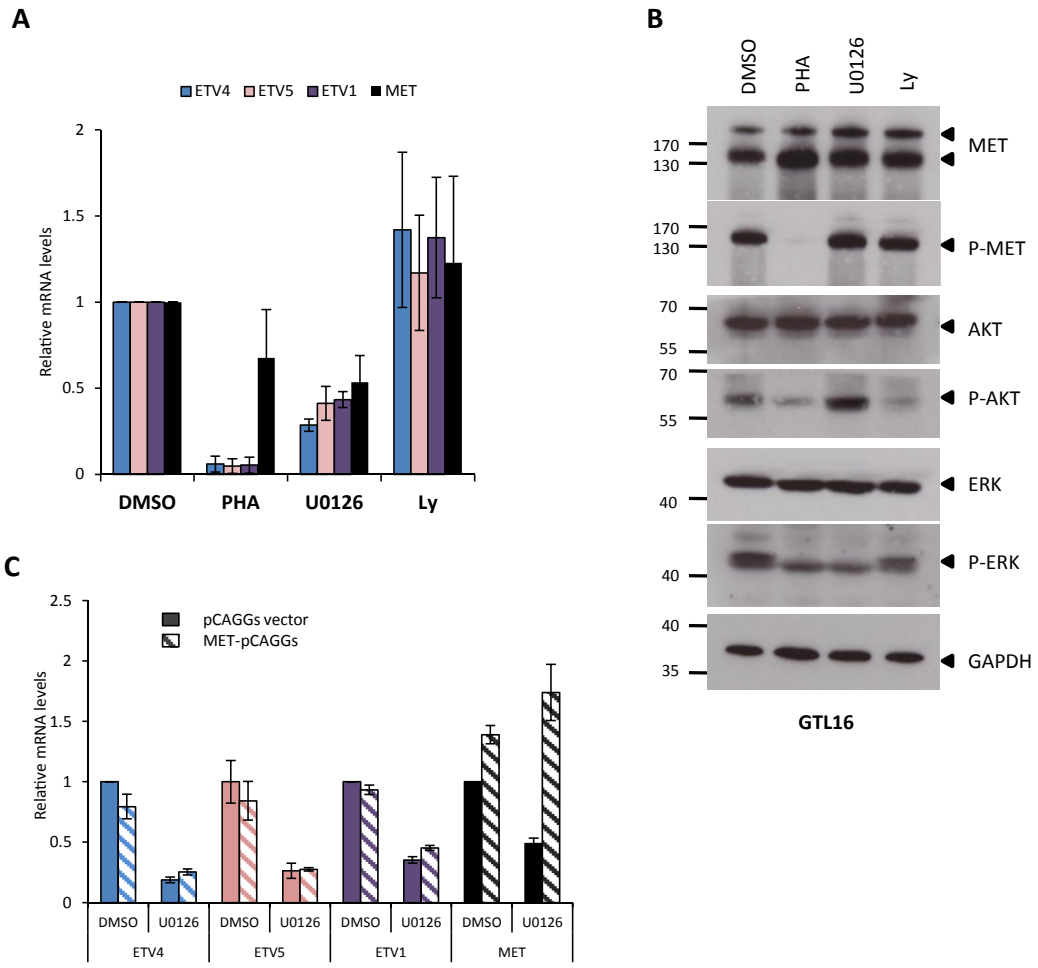

Figure 3 – Consequence of MEK and PI3K inhibition on PEA3 factors expression. (A) Expression levels of ETV4, ETV5, ETV1 and Met mRNA were measured by RT-qPCR in GTL16 after treatment 24 h with 0.4 μM of Met kinase inhibitor PHA, 10 μM of MEK inhibitor UO126 or 10 μM of PI3K inhibitor LY294002. (B) After similar above treatments, cells were harvested for Western analysis using antibodies directed against Met, phospho-Met, Akt, phospho-Akt, ERK, phospho-ERK and GAPDH to assess the loading. Arrow heads indicate positions of the proteins targeted by the respective antibodies. (C) GTL16 cells were transiently transfected with the pCAGGs empty vector or expressing full length human Met (Met-pCAGGs). The day later cells were treated with DMSO or U0126 for 24 h. Expression levels of ETV4, ETV5, ETV1 and Met mRNA were then measured by RT-qPCR.

growth is abrogated by Met activity inhibition (Corso et al., 2008). The Met addicted cells display several transformed phenotypes including growth factor independent proliferation, anchorage-independent growth, increase migration and invasion and resistance to cell death, all these responses being dependent on the receptor activity. Regulation of gene expressions is the final step required for most of the biological responses induced by Met. Consistently, the downstream signaling of the receptor is able to regulate the expression or the activity of several transcription factors, as for example STAT3, p53 or ETS family members (Furlan et al., 2012; Kermorgant and Parker, 2008; Paumelle et al., 2002). However, integration of the Met downstream signaling at the transcriptional level remains poorly understood. For instance, specific involvement of the transcription factors in the large panel of biological responses induced by Met is largely unknown.

We show here that, in Met addicted cells, expression of PEA3 transcription factors ETV1, ETV4 and ETV5 is dependent on Met activity. While PEA3 factors belong to a larger family including other ETS factors, only the expression of these three PEA3 factors was strongly dependent on Met, demonstrating that this restricted ETS subgroup is highly regulated by the receptor (Figure 8). Interestingly, two recent studies evaluating mRNA expression by high throughput strategy found that Met TKI inhibitors decrease PEA3 factors expression in cellular models displaying Met receptor addiction (Bertotti et al., 2009; Lai et al., 2014). Amplification of *met* gene was also recently observed in the context of resistance to tyrosine kinase inhibitors and monoclonal antibodies directed against EGFR in lung and colorectal carcinoma, respectively (Bardelli et al., 2013; Engelman et al., 2007). We demonstrate here that, in a lung cancer carcinoma cell line harboring a mutated EGFR,

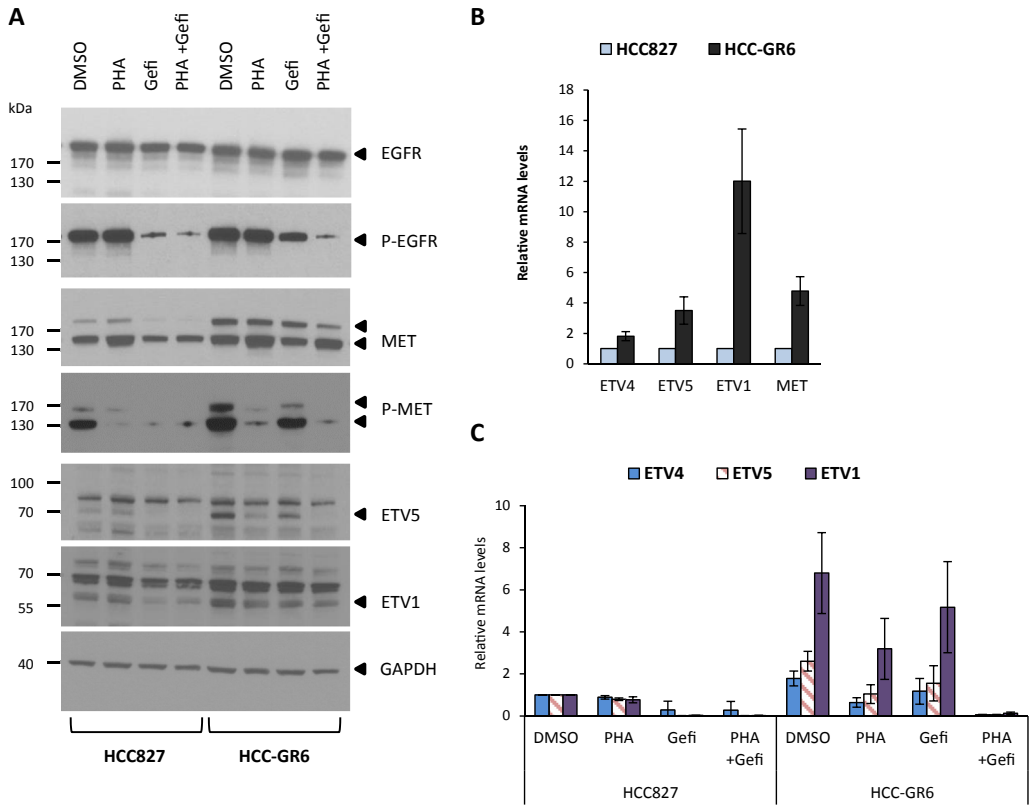

Figure 4 – Expression of PEA3 factors are under the control of both EGFR and Met activities in EGFR TKI resistant cells. (A, C) HCC827 and HCC-GR6 cells were treated 24 h with 0.4 μM PHA-665752 (PHA), 0.4 μM gefitinib (Gefi) or both inhibitors. (A) Cells were harvested for Western analysis using antibodies directed against EGFR, Phospho-EGFR, Met, phospho-Met, ETV5, ETV1 and GAPDH to assess the loading. (B, C)mRNA expressions of ETV4, ETV5, ETV1 and Met were measured by quantitative RT-PCR (RT-qPCR).

expression of the three PEA3 factors is dependent on EGFR activity. In the derived cells displaying resistance to TKI EGFR through *met* gene amplification, expression of PEA3 factors was increased and is dependent on both Met and EGFR. Furthermore, in cell line displaying EGFR mutation (HCC827) or *met* gene amplification (GTL16), the decrease of PEA3 factors expression induced by EGFR or MET tyrosine kinase inhibitors could be rescued by HGF or EGF treatment, respectively. This demonstrates that both receptor tyrosine kinases are able to regulate PEA3 factors expression, with ligand activation of each one able to rescue decrease of PEA3 expression induced by inhibition of the other.

Met and EGFR downstream signals share common main signaling pathways including RAS–RAF–MEK–ERK and PI3K–AKT (Bertotti et al., 2009; Wagner et al., 2013). These common signaling features were proposed to explain the efficient replacement of EGFR signaling by those of Met in the case of resistance. Accordingly, in cells harboring *met* gene amplification, we show that PEA3 expression is dependent on the activity of the kinase MEK of the RAS–ERK signaling pathway.

In Met addicted cells, inhibition of the receptor abrogated cellular proliferation and finally led to cell death. Interestingly, concomitant silencing of the three PEA3 factors did not lead to inhibition of proliferation nor survival or anchorage-independent growth, demonstrating that PEA3 silencing is unable to recapitulate Met inhibition. However, PEA3 silencing leads to efficient decrease of migration through a filter, individual cell displacement and invasion through Matrigel. Therefore, the three PEA3 factors are specifically involved in migration triggered by Met in transformed cell lines. This is consistent with several studies demonstrated that in various cell lines the PEA3 factors can regulate migration and invasion. For instance, we showed that in normal mammary epithelial cell lines, overexpression of ETV4 and/or ETV5 increase migration and invasion, while in metastatic mammary cell lines, its silencing prevent both responses (Chotteau-Lelievre et al., 2003; Firlej et al., 2008; Ladam et al., 2013) Other data reported that inhibition of ETV1, ETV4 or ETV5 expression in cancerous cells induces an inhibition of invasion and/or migration properties, with or without affecting cellular proliferation (Clementz et al., 2011; Hollenhorst et al., 2011; Yuen et al., 2011). In most of the cases, these effects are associated with the downstream regulation of MMPs, as for example MMP2 (Monge et al., 2007) or markers of epithelial to mesenchymal transition (Pellecchia et al., 2012), all hallmarks of tumorigenic emergence and progression. It was notably the case for gastric or lung cancer cells, in which PEA3 group members and ETV4, in particular, have been associated to tumorigenic properties (Hakuma et al., 2005; Hiroumi

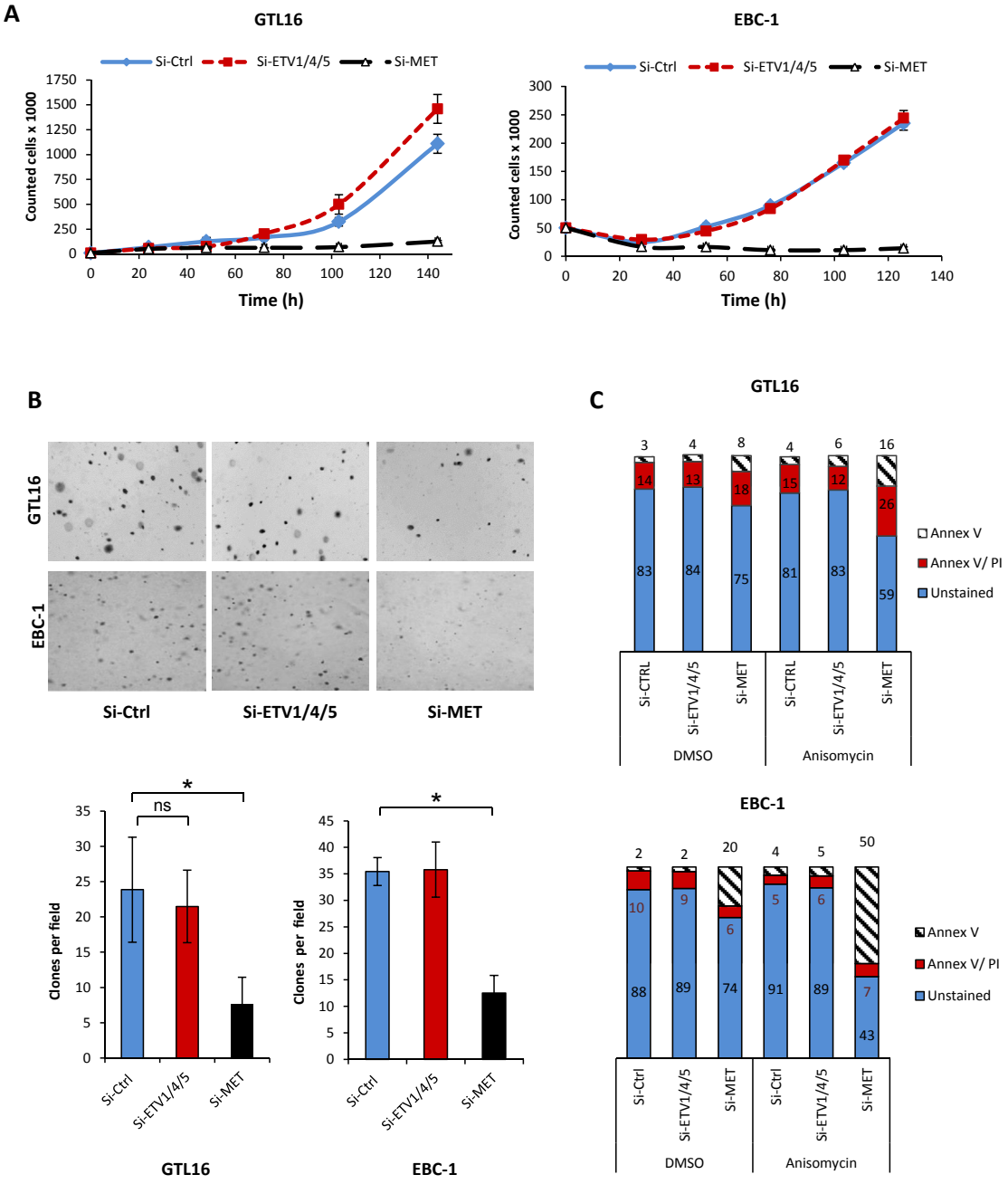

Figure 5 – Consequence of PEA3 knockdown on proliferation, anchorage independent growth and survival. (A, B, C) GTL16 and EBC-1 cells were transfected with control siRNA (Si-Ctrl) or siRNA targeted ETV1, ETV4 and ETV5 or Met. (A) Proliferation of the GTL16 and EBC-1 was determined by cell counting during 5 days (n = 3; ±SD and results are representative of three independent experiments). (B) Anchorage independent growth was measured by counting clone formation in soft agar after 10 days. Representative pictures of fields are shown. The quantification presented is the average of clones counting from three independent experiments; * = p-value < 0.05. Other comparisons were not significant. (C) GTL16 and EBC-1, silenced for ETV1, ETV4 and ETV5 or Met, were treated or not 4 h with 50 μM anisomycin. Apoptotic cell death was evaluated by Annexin V (annex V) and Propidium Iodide (PI) staining. Representative experiment showing percentage of unstained, Annexin V and PI staining, is presented.

et al., 2001; Keld et al., 2011; Li et al., 2011; Meng et al., 2012; Yamamoto et al., 2004). Moreover, ETV4, coupled with an active ERK signaling, has been associated with a poor prognosis in gastric carcinoma (Keld et al., 2011). In colorectal carcinoma, ETV4 overexpression was associated to the promotion of invasive and metastatic potential by affecting invasion but not proliferative capacities and deregulation of MMPs expression (Mesci et al., 2014). Similarly, ETV1 overexpression in human LNCaP prostate cancer cells did not influence proliferation, but favored cell migration through MMP7 transactivation (Shin et al., 2013). Our data are in line with these reports regarding migration and proliferation events

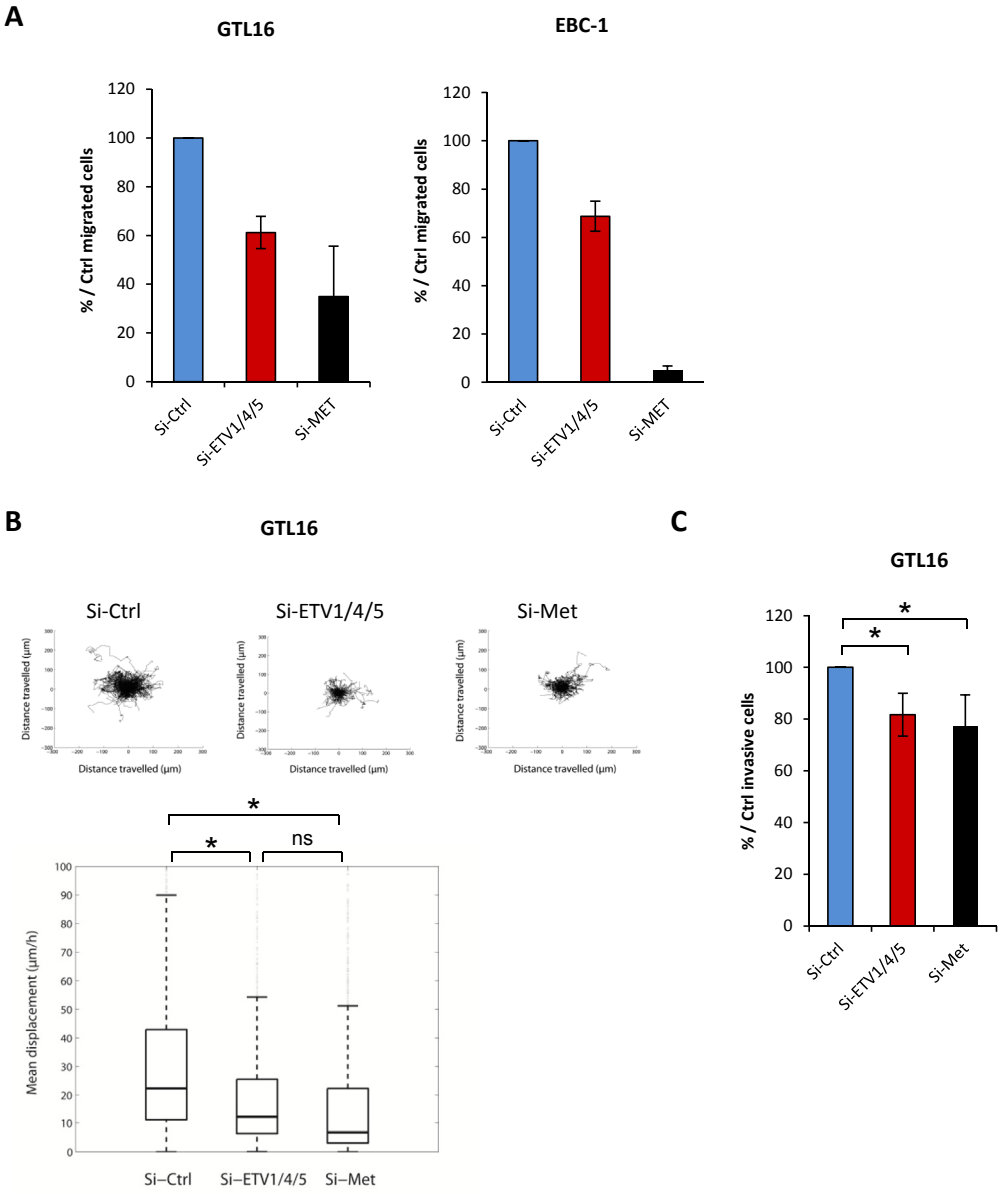

Figure 6 – Consequence of PEA3 knockdown on migration and invasion. (A, B, C) GTL16 and EBC-1 cells were transfected with control siRNA (Si-Ctrl) or siRNA targeted ETV1, ETV4 and ETV5 or Met. (A) Migration of GTL16 and EBC-1 was determined using Boyden transwell chambers. Cells migrating to the bottom surface of the transwell were fixed, stained and counted. The results presented are an average of seven wells from three independent experiments (±SD). (B) GTL16 were stained with fluorescent Dil-C12 and seeded at low density. Five hundred trajectories of individual cells were tracked by fluorescent video microscopy. Velocity of cells in µm/h was shown in a boxplot; * = p-value <0.05. (C) GTL16 cells invasion was determined using Fluoroblok modified Boyden chambers coated with Matrigel. The fluorescence of Dil-C12 labeled-cells migrating to the bottom surface of the transwell was quantified and data were expressed as a percentage with respect to that of the control siRNA, set to 100%. The results presented are an average of three independent experiments; * = p-value <0.05.

and highlighted the specific deregulation of MMP2 expression in Met-amplified tumor cells.

Redundancy of PEA3 factors have been demonstrated *in vivo*. Indeed, knockout mouse models inactivated for four alleles of both ETV4 and ETV5 failed to develop kidney, while indifferent deletion of three or two alleles of ETV4 or ETV5 resulted in less severe phenotype (Lu et al., 2009). Thus, during development both factors seem to act together to regulate epithelial organ morphogenesis. By contrast, ETV1 knockout revealed that this factor plays an important role in motor co-ordination, an involvement drastically different from the two other PEA3 factors (Arber et al., 2000; Kucera et al., 2002). *In vitro* DNA binding assays demonstrate that among the ETS transcription factor family, the PEA3 factors preferably bind similar DNA sequence (Wei et al., 2010). Interestingly, in the Met-amplified cellular models, efficient inhibition of

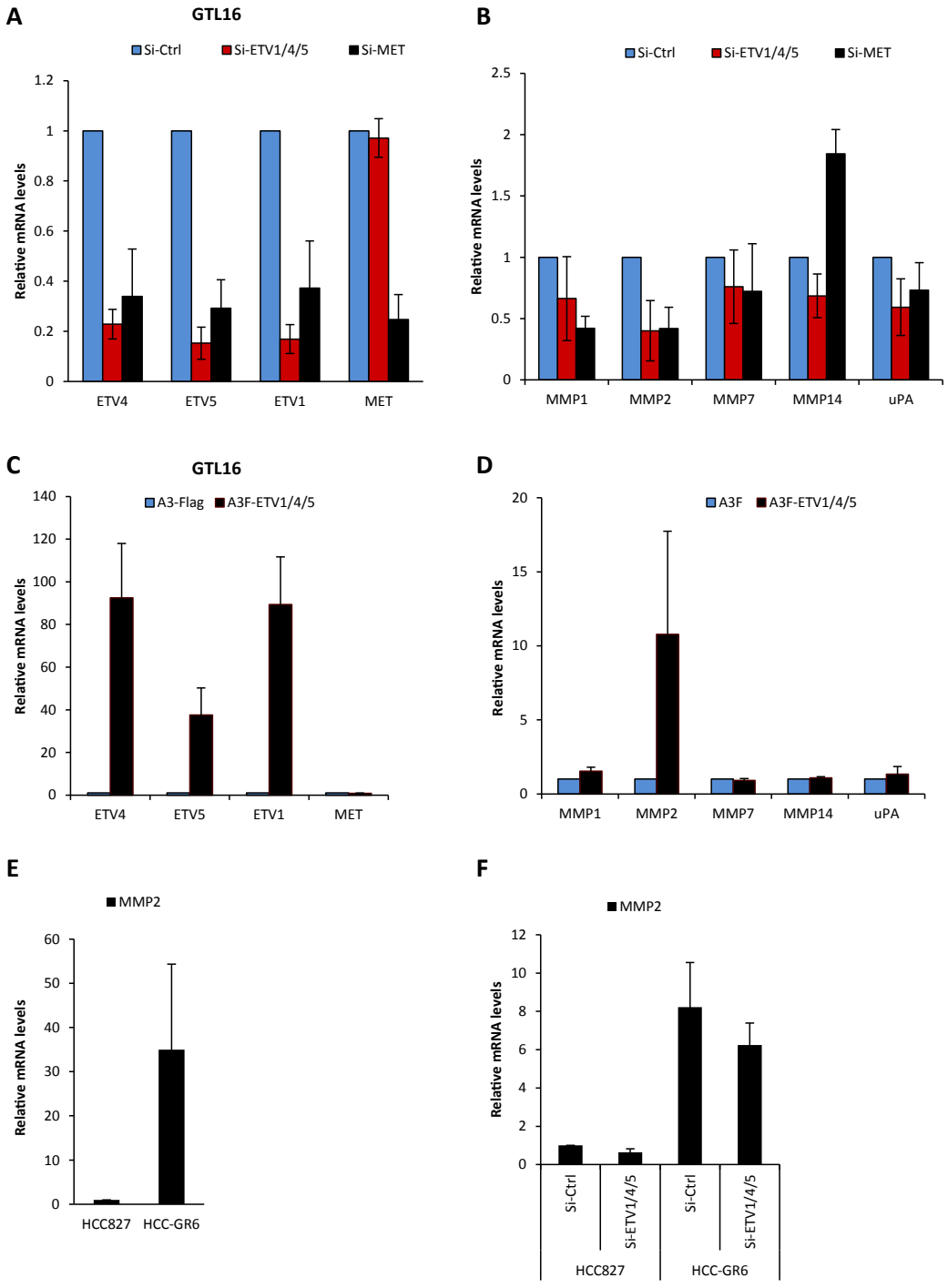

Figure 7 – MMP2 expression in Met and PEA3 factors silenced cancer cells. (A) GTL16 cells were transfected with control siRNA (Si-Ctrl) or siRNA targeted ETV1, 4 and 5 or Met. RNA were prepared 48 h after transfection and expression of the PEA3 factors and Met mRNA were quantified by RT-qPCR. (B) mRNA expression of MMP1, 2, 7, 14 and uPA was detected using RT-qPCR after PEA3 or Met knockdown by specific siRNA in GTL16 cells. (C) Expression of PEA3 factors and Met was determined in GTL16 cells transfected with empty vector (A3-Flag) or with vectors expressing Flag-tagged ETV4, ETV5 or ETV1. (D) In these transfected cells, MMP1, 2, 7 and 14 and uPA expressions were determined. (E) MMP2 mRNA levels between HCC827 and HCC-GR6 were compared. (E) PEA3 factors expression was silenced in HCC827 and HCC-GR6 cells and relative MMP2 mRNA level was determined by RT-qPCR.

migration and invasion is achieved only when concomitant silencing of the three members of the PEA3 subgroup were performed, with only weak and not significant inhibition when each one is silenced (data not shown). This suggests that in Met-addicted situation, the three PEA3 factors are redundant and act all together to control migration and invasion. Therefore, in Met addicted cells where the PEA3 factors are overexpressed compared to other cancerous cells, this

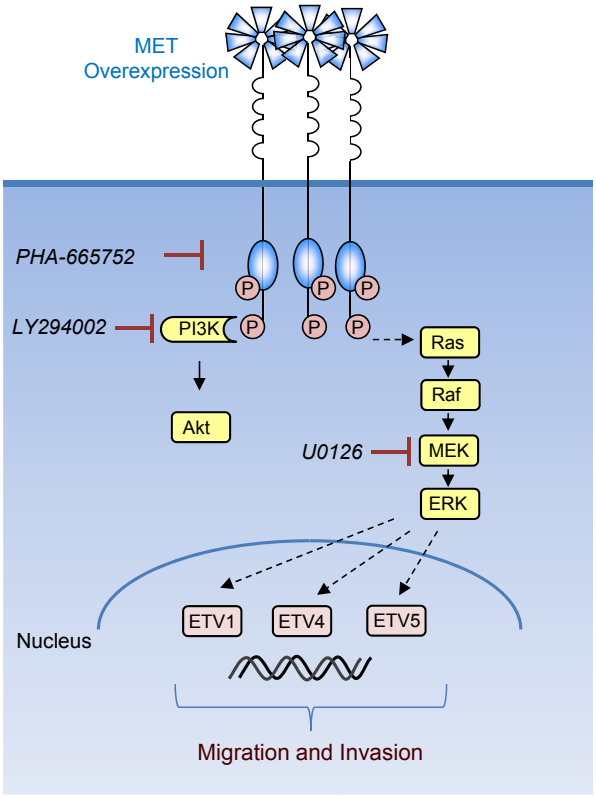

Figure 8 – Schematic representation of the Met receptor and its downstream signaling pathways regulating expression the PEA3 factors ETV1, 4 and 5 involved in migration and invasion. Pharmacologic inhibitors and theirs targets used in the study are noted.

overexpression could lead to a less tightly regulation of target genes leading to undifferentiated action of each factor.

Expression of PEA3 factors can be regulated by gene amplification of other receptors. Indeed, in breast cancers, PEA3 mRNA expressions are increased in tumors harboring HER2/neu gene amplification which is found in about 20% of the cases (Benz et al., 1997). Furthermore, HER2 can promote transcriptional activity of PEA3 factors through their phosphorylation on serine residues induced by ERK, demonstrating that HER2 can regulate this subgroup of factors through both increase of their expression and transcriptional activity (Bosc et al., 2001; O'Hagan et al., 1996). Finally, expression of a dominant negative from of PEA3 in transgenic mice inhibits HER2-induced tumorigenesis (Shepherd et al., 2001). Because Met triggers efficient activation of the RAS–ERK signaling, it could increase as well the transcriptional activity of the PEA3 factors. Taken together, several RTK displaying ligand-independent activation can regulate expression or activity of PEA3 factors through their common RAS–ERK downstream signaling.

Beside PEA3 factors, other ETS transcription factors are partially dependent of Met activity in both GTL16 and EBC-1 cells lines such as ETS1, ETS2 and NET, displaying about 50% of mRNA decrease upon Met inhibition. By contrast, ESE2 (ELF5) transcription factor expression displays more than three times increased upon Met inhibition. Interestingly, using ESE2 knockout experiments, Chakrabarti and colleagues demonstrated that ESE2 is able to suppress epithelial–mesenchymal transition and reduce cell migration, tumor growth and metastasis in mouse mammary glands (Chakrabarti et al., 2012). We can speculate that in addition to increase expression of the PEA3 factors, Met signaling could repress ESE2 expression to favor migration.

Overexpression of Met was found in about 50% of patients with lung and gastric cancers. However, Met overexpression does not seem to be predictive factor for the developed Met targeted therapies. Indeed, two recent phase III clinical trials evaluating a tyrosine kinase inhibitor (TKI) and a monoclonal antibody against Met in non-small cell lung cancer (NSCLC) failed to demonstrate efficiency on this large patient subgroup displaying Met overexpression. However, Met overexpression monitored by immunochemistry does not necessarily reflect Met activation in these tumors, thus leading to an overestimation of likely responders. By contrast, results of ongoing clinical trials suggest that *met* gene amplification could be a better predictive biomarker. Indeed, TKI against Met induced an objective response rate in about two thirds of this restricted subgroup of patients with NSCLC and gastric cancers displaying *met* gene amplification (Camidge et al., 2014; Hong et al., 2014). Since, we demonstrate here that PEA3 factors are required for efficient migration and invasion of transformed cell lines displaying *met* gene amplification, the targeting of their transcriptional activity or target genes could be an alternate strategy to inhibit Met dependent tumorigenesis.

Acknowledgments

We would like to thank M. Duterque-Coquillaud and N. Malaquin for providing several primers and antibodies; Professor P.A. Jänne for providing HCC827 and HCC-GR6 cell lines; J.L. Baert, A. Verger and E. Lelievre for helpful discussions.

This work was supported by the CNRS, the Institut Pasteur de Lille, and INSERM, and by grants from the "Ligue contre le Cancer, comité Nord et comité Aisne", the "Association pour la Recherche sur le Cancer", the "Institut National du Cancer (PLBIO 2012 – DA N°2012-116)", the "Cancéropôle Nord-Ouest" and the SIRIC ONCOLille. We thank the Microscopy-Imaging-Cytometry Facility of the BioImaging Center Lille Nord-de-France for access to instruments and technical advice.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.molonc.2015.07.001.

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